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Vol. XLVI *March, 1924* *No. 3*

BIOLOGICAL BULLETIN

LUTEAL CELLS IN THE GONAD OF THE PHALAROPE.

HARRY B. YOCOM.

DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF OREGON.

It has been shown by Pearl and Boring (1917, 1918) that there are in the ovary of the domestic fowl certain interstitial cells which are in many ways similar to, and which they claim are homoiogous with the corpus luteum in mammals. A thorough study has convinced the authors that these cells are a constant element in the ovary of all fowls but that they are not found in the testes of normal male fowls.

Boring and Morgan (1918) made a study of the gonads of both male and female Sebright Bantams, a race of fowls in which the males and females are feathered alike. They found in both ovary and testis groups of cells like those described by Pearl and Boring as luteal cells, and ascribed to these so-called luteal cells the function of influencing the type of feathering. Since luteal cells are found in the ovaries of normal females or hen-feathered fowls and not in the testes of normal males or cock-feathered birds, but in the testes of hen-feathered Sebright Bantams, they reason that the normal feathering of fowls would be of the male type were it not for the suppressing influence of the hormones secreted by the luteal cells. Further proof in favor of the contention is obtained from the well known facts of castration where hen-feathered females assume typical cock-feathers upon the removal of the ovary, and that the hen-feathered male Sebright becomes cock-feathered after the complete removal of the testes.

It has seemed that further evidence concerning the function of luteal cells might be brought to bear if a histological study were made of the gonads of birds other than fowls, and especially some bird in which the sex differences are reversed; that is some race of

birds in which the females are more brilliantly colored than the males. Such a sex difference is characteristic of the Phalarope. During the spring migration when the birds are in full breeding plumage the females are easily distinguished from the males by the more brilliant plumage of the back and neck. Bailey (1917) states the sex differences as follows: "Male in breeding plumage; upper parts dark plumbeous, striped on back with buff and black: sides of neck rufous, chest gray, upper throat and belly white. Female in breeding plumage; brighter colored, rufous extending across throat as well as on sides of neck." To one not acquainted with the facts concerning the sex differences of this group of birds the sexes would undoubtedly be mistaken. During the fall migration both males and females are lighter in color due to the loss of the rufous color and more nearly resemble one another.

In one respect the sex differences of the Phalarope do not compare with the sex differences of the fowls and that is in the fact that the color differences in the Phalarope are not associated with structural differences in the feathers, as is usually the case in the fowls. A microscopical examination of the feathers of the Phalarope showed that the color was due to pigment and not to a difference in structure. Whether or not this is an important difference and alters our problem can be determined only by a study of a large number of birds or different races.

The material with which this study has to deal consists of the gonads of both male and female Northern Phalarope, *Phalaropus lobatus*, Linn., collected during both fall and spring migrations along the Oregon coast. The tissues were taken from the birds as soon as possible after they had been shot, and were put into Bouin's fluid and left until it was convenient to care for them in the laboratory. Delafield's hæmatoxylin was found satisfactory and was used almost exclusively as a stain.

A study of the testes gave only negative results. In no case were there found any groups of cells resembling the characteristic packets of luteal cells found in the testes of the Sebright, and not even single cells which might be considered of that type. The intertubular material was not abundant and consisted of narrow spindle shaped cells with oval, more or less shrunken nuclei.

In the ovary, however, characteristic groups of the so-called luteal cells were found in the theca surrounding the oöcyte follicles

Figs. 1 and 2). These groups of cells are present in the material collected in May and September but the groups of cells seem to

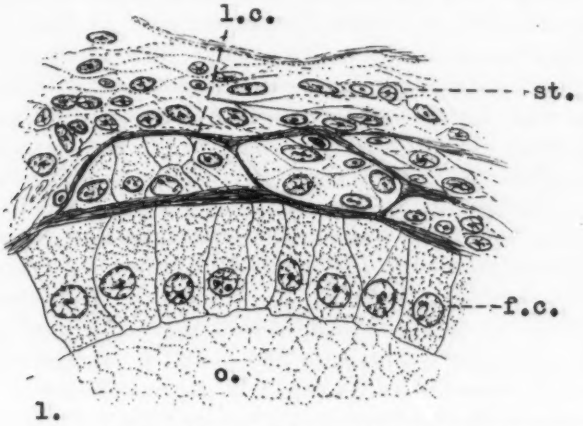

Fig. 1. Section of ovary of Phalarope in fall plumage. × 1000.

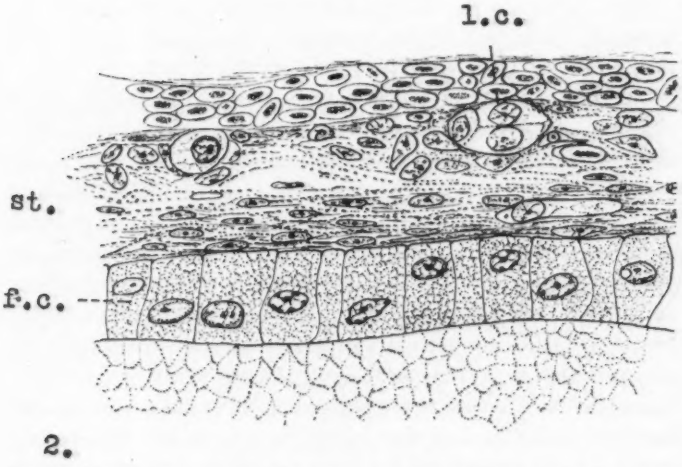

Fig. 2. Section of ovary of Phalarope in spring plumage. × 1000. *f. c.*, follicle cells, *l. c.*, luteal cells, *o.*, oöcyte, *st.*, stroma.

be somewhat larger and more clearly defined in the material collected in the fall when the sexes were more nearly alike as regards feathering. In no case could it be said that the groups of

luteal cells were numerous. In many sections there would be no packets at all while in others there would be one or two. In all cases where found the packets were clearly defined and easily demonstrable, being composed of cells having a relatively large amount of cytoplasm with rounded nuclei. The cytoplasm of the luteal cells takes very little of the hæmatoxylin stain. In this respect as well as in shape and size these cells differ from the cells of the stroma of the ovary.

The facts cited above do not indicate that luteal cells are in any way influential in suppressing color development in the Phalarope. In their consideration of the influence of the luteal cells on feathering in fowls Boring and Morgan have not separated the factors for color and structure in the feathers. In the present study only the color of the feathers is considered. The fact that luteal cells are found in the ovary while the female is so much more brilliantly colored seems to eliminate the possibility that the secretions of the luteal cells in any way suppress color development.

The evidence so far obtained is not sufficient to warrant any statement in regard to the problem of the association of color differences with structural differences in the feathers.

The recent work of Nonidez (1922) raises another question the solution of which will in a large measure depend on a more extensive study of the presence or absence of luteal cells in the gonads of birds. This author has shown that the so-called luteal cells as designated by Pearl and Boring and Boring and Morgan are in reality only the degenerating sex cords of the embryonic gonad. What have been termed luteal cells and what have been understood to be homologous with the corpus luteum of mammals, Nonidez has shown to have an entirely different origin and are therefore not to be confused in terminology with the mammalian tissues. Whether or not these remains of the sex cords may have an endocrine function is left by the author as an open question to be determined by a wider study of a variety of material. The facts as presented above as regards the presence of these cells in the gonads of the Phalarope do not seem to bring us any nearer a solution of the problem. There is no indication, in the Phalarope, that these groups of cells exert a suppressing influence on the development of the color of the feathers of the

two sexes. That they may have some other endocrine function, in the light of our present knowledge, is to be neither affirmed nor denied.

Conclusions.

A histological study of the gonads of the Northern Phalarope shows that luteal cells such as are found in the gonads of Sebright Bantams are absent in the testes and present in the ovaries. Since the female is more brilliantly colored than the male there is no indication that these cells through an internal secretion influence the development of any differences in the color of feathers of the two sexes.

Literature.

Bailey, F. M.

'17 Handbook of Birds of the Western United States. Pp. lii + 574, 601 figures.

Boring, A. M., and Pearl, R.

'17 Sex Studies. II. Interstitial cells in the reproductive organs of the chicken. Anat. Rec., **13**, 253–268, 6 figs. in text.

Boring and Morgan, T. H.

'18 Luteal cells and hen-feathering. Journ. Gen. Phys. **1**, 127–131, 4 figs. in text.

Nonidez, Jose F.

'22 Studies on the Gonads of Fowls. III. The origin of the so-called luteal cells in the testis of hen-feathered cocks. Am. Jour. Anat., **31**, 109–124, 7 figs. in text.

Pearl, R. and Boring, A. M.

'18 Sex Studies. X. The corpus luteum in the ovary of the domestic fowl. Am. Journ. Anat. **23**, 1–36, 9 pls., 6 figs. in text.

INTERACTIONS OF PROTOPLASMIC MASSES IN RELATION TO THE STUDY OF HEREDITY AND ENVIRONMENT IN *ARCELLA POLYPORA*.[1]

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INTRODUCTION.

In the first article of this series, Kepner and Reynolds ('23) have shown that under favorable conditions *Difflugia* usually reappropriates, by fusion, fragments of protoplasm which have been severed from the cell-body. In the last paragraph of their summary these authors make the following statement: "Observations have been made in which individuals of the same species obtained from the same wild culture, showed a decidedly negative response towards each other's fragments, yet in other instances such cross-fusions did occur." Elsewhere in their paper two suggestions are made as to the probable explanation of why some specimens did fuse with fragments belonging to other individuals, viz: "(*a*) *The individuals were closely related by having a recent common ancestor.* (*b*) *By living in the same surroundings, the environmental influences have acted upon both organisms in such a way as to cause an identical physiological state.*" The question thus raised seemed to be of sufficient importance to justify further work on the subject; consequently the experiments incorporated in this paper were conducted for the purpose of clarifying the situation.

The previous publication was largely concerned with describing the process of protoplasmic fusion. Although a number of observations were made upon the reactions between cell-bodies and fragments from other individuals of the same species, no attempt was made to culture the organisms and then test for reactions between members of a clone. As a result, only two

[1] This is the second article of a series dealing with the phenomenon of fusion between protoplasmic masses. The first article was written by Wm. A. Kepner and B. D. Reynolds ('23) from the University of Virginia. Further work on the subject is now in progress.

types of reactions were observed: (*a*) fusion and (*b*) failure to attract, or if contact was made the fragment would be pushed along for a while, without causing any apparent disturbance, and then abandoned.

During the course of this investigation, involving much more extensive experiments, it was found that in some cases the masses would readily attract [1] each other, but upon making contact there would be a sudden contraction of the involved regions as if violently shocked, while the protoplasm constituting both the fragment and the organism's pseudopod (*s*) which had made contact with it would be shattered into bead-like masses. See Fig. 1: *A*, *B*, and *C*. This type of reaction was observed so frequently that it is referred to throughout this paper as the "shattering" reaction.

As the investigation progressed it seemed advisable to divide the problem into three sections; and to study the reactions between organisms and fragments from related specimens under the influence of three different conditions as follows:

I. Similar environments.
II. Different environments.
III. Identical environments.[2]

Numerous methods have been employed in studying variations among the protozoa, such as: rate of reproduction, measurements of size, counting the number of spines, teeth, tentacles, etc., resistance to poisons and to high temperatures. In this study, for the first time, advantage has been taken of the property possessed by certain organisms of fusing with their own or fragments from closely related specimens. The superiority of this method lies in the fact that it affords a quick and definite means of determining

[1] In this, as in the previous paper, it was not determined why two such masses were attracted to each other. When protoplasmic fragments were not present the organisms would move about at random; but when pieces of protoplasm which had been detached from themselves or from related individuals were within two or three hundred micra of them, they would invariably proceed directly towards the fragments. This statement is based on over fifteen hundred observations. A series of experiments are being planned to ascertain, if possible, the stimulus involved in these positive reactions.

[2] The organisms comprising the two lines under observation were placed in the same concavity, or else, portions of the culture media were frequently exchanged between the concavities containing them.

minute physiological differences—even before these changes have become expressed morphologically. Furthermore, the physiological difference between two organisms, as indicated by this method, appears to be more permanent and less subject to fluctuations than certain morphological variations which have been utilized by others. In some instances cross-fusion was observed to take place readily between specimens belonging to the same clone—though they appeared quite different to the eye. In other words: the physiological variations, as determined by means of the ability or inability of an individual to fuse with a fragment of protoplasm belonging to another, seem to be independent of differences in size or shape of the two animals concerned.

Fusion between cell-bodies and severed fragments has been observed by the author in ten species of the genus *Difflugia*, in *Arcella polypora*, *Centropyxis aculeata*, and also other forms of protozoa. The phenomenon seems to be fairly common among the thecamœbæ. Perhaps it would be well to emphasize at this point the fact that throughout these experiments any individual *Arcella* would fuse with its own fragments (provided they were not too far removed, or had not been separated for too long a period) under any environmental conditions to which they were subjected.

ACKNOWLEDGMENTS.

This problem suggested itself to me while I was discussing with Professor Wm. A. Kepner, the results of our work ('23) *Reactions between Cell-bodies and Pseudopodial Fragments of Difflugia*. I wish to express my deepest gratitude to Professor R. W. Hegner and Dr. Wm. H. Taliaferro, under whose direction the work was done, for their valuable advice and criticisms. I am also deeply indebted to Professor H. S. Jennings for reading the manuscript and making some important suggestions.

THE ORGANISM.

The experimental animal used in this investigation was a multinucleated *Arcella* closely resembling the species described by Penard ('85) as *A. polypora*. Hegner ('20) worked with the same organism, calling it *A. polypora*. However, in the same paper he also gave measurements of a larger multinucleated form which

agrees with the amended description of *A. polypora* given by Penard ('92) viz: "*très grande, à bouche très largement ouverte, et toujours pourvue de noyaux en nombre considerable.*" Apparently Hegner is the only investigator in this country who has reported the existence of *Arcellae* containing many nuclei. Leidy ('79) states that he was able to detect two, occasionally one, and rarely three, though admitting that he had possibly overlooked the presence of some nuclei in the larger forms of *Arcella*. Occasionally large, polynucleated, wide-mouthed specimens were found in my collections. The differences between these two multinucleated forms seem to be great enough to justify the creation of a new species. However, if this is done, the law of priority makes it necessary to retain the name *A. polypora* for the smaller form which was used in these experiments.

Because of the existing confusion it is deemed advisable to say a few words regarding certain characteristics possessed by the organism studied: shape, structure, and coloration of shell similar to *A. discoides;* diameter of shell from 80 to 120 micra; height of shell from 25 to 40 micra; oral orifice approximately equivalent to one third the diameter of shell; number of nuclei usually eight or ten. Careful scrutiny under the oil immersion failed to reveal any minute pores around the mouth of the shell, but the shadows at the outer margins of the large cancelli lining the buccal aperture might readily be misinterpreted as such under a low magnification.

Arcella polypora was selected as the object for study because it reproduces rapidly, is easy to culture, and is capable of withstanding the influences of various stimuli. This rhizopod may be found in either large or small bodies of water, living especially on the under surfaces of aquatic plants, in decaying vegetable matter, and ooze. On April 10th. 1922 a collection, containing many *A. polypora*, was obtained from an artificial pool in the botanical garden near the Johns Hopkins Biological Laboratory. All of the organisms used in these experiments were descendants of this lot. A mass culture was kept going, and as new individuals were needed they were isolated and new clones were started from them.

METHODS.

(*a*) *Apparatus and Technique.*

In carrying out these experiments more standardized and exact methods were employed than those used in the first article of this series, though they were essentially the same. In every case (unless specified) distilled water (pH = 0.7) was used, and each animal was washed once or twice before being transferred to the slide on which the amputations were made. Furthermore, any detritus clinging to the shell was removed before the operation. In all of the cross-fusion experiments the two organisms to be tested were placed on the same slide, and when a fragment was severed from one, that individual was moved away and the other placed near the fragment of protoplasm. The glass needles, with which the protoplasmic masses were detached, were drawn from standard Pyrex tubing $\frac{1}{4}$ OD furnished by the Corning Glass Company, Corning, N. Y. All operations were performed under the compound microscope—32 mm. objective and No. 10 eyepiece (B. & L.)—while the 16 mm. objective was used in making the observations. A camera lucida was attached to the microscope and at intervals (usually one minute) the outlines of the objects were traced with a pencil. These sketches were 170 times the size of the objects.

The pseudopods of *A. polypora* are usually digitate and rarely extend far beyond the periphery of the shell, making it very difficult to sever masses of protoplasm by cutting them off with a needle as was done in the case of *Difflugia*. This difficulty was overcome by taking advantage of two things—(*a*) the shape of the shell, and (*b*) the fact that if undisturbed for a while the animal ordinarily attaches itself to the substratum. The organism is shaped somewhat like a shallow bowl, the outer rim of the shell forming a rather sharp edge, Fig. 2. If, after a pseudopod has been extended beyond the outer margin, pressure is applied to the top of the shell the pseudopod will be caught between the rim and the sub-stratum and its distal end will thus be pinched off. Severance is aided by the animal's sudden reaction to this stimulus. Sometimes a specimen would fail to protrude pseudopods of sufficient length to permit use of the method just described; consequently it was left undisturbed for a few min-

utes and then suddenly displaced by means of a glass filament. This procedure would frequently result in a large mass of protoplasm being torn from the organism.

(*b*) *Culture.*

Hollow-ground slides, each containing two concavities, were used as receptacles in cultivating the organisms. Seven drops of the desired culture medium were put into each concavity and the animals were transferred to them. After this the slides were labelled and placed in transparent glass moist chambers which were kept before a window, unless otherwise stated.

At first the method described by Jennings ('16) for *Difflugia* was used in preparing the culture medium, *i.e.*, a quantity of ooze from a pond in which the organisms were found to be living was shaken up in water, this was allowed to settle, and then the water was decanted off and used. Although other investigators have obtained successful results by using culture media prepared in this way, several objections to its use in these experiments were evident: first, fresh material was hard to obtain, since no ponds were near the laboratory in which the work was done; secondly, a constant culture medium was not insured, for the plant and animal life in a given pond vary constantly; and finaliy, it was necessary ever to be on guard against possible contamination with wild specimens and injurious chemical agents. After encountering these difficulties for two months the above method was discarded in favor of a hay infusion culture medium which dispensed with all of the objections mentioned, and in which the organisms thrived even better. It was prepared as follows: 10 grams of clean timothy hay was placed in a clean beaker containing 250 c.c. of neutral distilled water. This was boiled slowly for five minutes, then strained through two thicknesses of cheese cloth, after which it was stored, in quantities of about 3 cc., in small sterile test tubes. The tubes were then plugged with cotton and placed in boiling water for fifteen minutes. Two days later they were subjected to boiling water again for the same period of time, in order to kill any bacteria which might have resisted the first sterilization by being in the spore stage. The liquid in the tubes constituted what was known as stock solution; it would keep for months without deteriorating

provided evaporation didn't take place. In making up the culture medium one part of the stock solution was taken and to it were added nine parts of distilled water—giving a 10 per cent hay infusion solution. When other ingredients such as sugar, alcohol, etc., were used, they were mixed with distilled water in the desired proportions and then nine parts of this were added to one part of the stock solution. Thus, in every case the percentage of hay infusion remained the same. After a tube containing some of the stock solution had been opened and a part of its contents used the remainder was discarded. The *Arcellæ* grown in this medium subsisted largely on bacteria, the progenitors of which were introduced with the organisms.

(*c*) *Pedigrees.*

"Pure" lines, or clones, were obtained by isolating specimens from the wild culture and designating them, for example, as the progenitors of clone *A*, clone *B*, etc. Upon dividing, the daughter-cell remaining in the old shell, and all of its descendents, were referred to as clone *A* line *a*, while the daughter-cell occupying the new shell, and all of its descendents, were denominated clone *A* line *ad*. Fig. 3 expresses graphically the procedure followed.

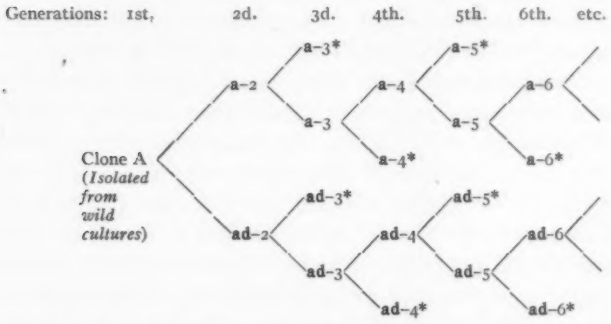

FIG. 3. Diagram illustrating method employed in recording the relationship of experimental animals. Those marked with an asterisk were either used for observations, or else discarded.

When a different environment was imposed on one line of a clone that line was designated, for instance, as clone *A* line **a-*su*** (*su* indicating that sucrose was added to the culture medium in

which this line was nourished). Ordinarily lines were perpetuated through the individuals remaining in the old shells, but this rule was not strictly adhered to since experience showed no difference in the results obtained when other members were used. From two to four individuals of each line were kept in culture as a precaution against possible fatalities.

I. Similar Environments.

These experiments are discussed under the heading "similar" environments because the external conditions surrounding the two lines of the clones involved were as nearly alike as it was possible for the experimenter to make them. Though kept apart from the first division of the clonal parent, the two diverging lines were treated in exactly the same manner; for instance: the same pipette was used in transferring members to fresh concavities; these concavities contained culture medium taken from the same bottle; afterwards the depression slides containing the organisms were placed alongside each other in the same moist chamber. Frequent tests were made, according to the method described above, between individuals of one line and fragments of protoplasm from individuals of the other. It was found that daughter-cells (members of the second generation) would readily fuse with each other's severed fragments—and that such reactions would occur regardless of whether or not the organism taking up the fragment had just previously lost a part of its own protoplasm. Furthermore, in every case when both lines were kept under similar conditions, cross-fusions continued to be exhibited for fifteen or more generations; consequently in later experiments tests for interactions were not begun until several generations had elapsed.

A series of experiments conducted between the two lines of clone I are given in extenso in Table I. It will be seen by referring to this table that cross-fusion between line **i** and line **id** persisted until the 22d day and 24th generation. After this no further inter-fusions occurred, but instead the protoplasms usually were shattered upon making contact. Attention should be called to the fact that in some of the experiments listed in Table I. the organism was on its back when placed near the fragment; in other cases the fragment would become detached from the sub-

TABLE I.

INTERACTIONS OF CELL-BODIES FROM ONE LINE AND PSEUDOPODIAL FRAGMENTS OF SPECIMENS FROM THE OTHER IN CLONE I, LINES i AND id. BOTH LINES WERE EXPOSED TO SIMILAR ENVIRONMENTS. 10 PER CENT. HAY INFUSION USED AS CULTURE MEDIUM. EXPERIMENTS WERE STARTED ON JUNE 25, AND ENDED ON JULY 31, 1922.

No. of Days Since Experiment was Begun.	Generation of Cell-body.	Generation of Fragment.	Horizontal Dimensions of Fragment in Micra.	Distance between Cell-body and Fragment: in Micra.	Time in Minutes before Contact was Made.	Reaction after Contact.
16	i-18	id-17	9 x 25	50	2	Fusion within 1′.
16	id-17	i-18	10 x 18	100	3	Immediate fusion.
16	i-18	id-17	15 x 40	110	3	Fusion after 3′.
18	i-20	id-19	12 x 15	90	–	No contact.
18	id-19	i-20	10 x 48	85	1.5	Fusion within 30″.
22	i-24	id-24	30 x 40	160	4	No fusion.
22	id-24	i-24	9 x 30	150	3	Violent contraction.
22	id-24	i-24	12 x 25	55	2	Shattering reaction.
25	i-28	id-27	8 x 38	95	–	No contact.
25	i-28	id-27	7 x 45	48	–	No contact.
25	id-27	i-28	45 x 55	32	1	Shattering reaction.
27	id-28	i-31	10 x 40	10	0.5	Shattering reaction.
29	i-34	id-32	14 x 20	82	2	No fusion.
29	id-32	i-34	12 x 30	15	3	Shattering reaction.
29	id-32	i-34	25 x 35	6	3	Shattering reaction.
32	i-38	id-36	20 x 30	62	–	No contact.
32	i-38	id-36	20 x 30	0	0	Slight shattering.
32	id-36	i-38	24 x 26	40	2	Slight shattering.
32	i-38	id-36	10 x 60	10	6	No fusion.
32	id-36	i-38	15 x 45	47	1.5	Shattering reaction.
32	i-38	id-36	8 x 25	0	0	No fusion.
36	i-43	id-41	7 x 70	78	2	Shattering reaction.
36	i-43	id-41	6 x 40	105	4	Shattering reaction.
36	id-41	i-43	20 x 60	53	1	Shattering reaction.

stratum and float away, or else be pushed along by the organism's pseudopods without making intimate contact with them. This accounts, to a great extent, for discrepancies observed in the time required to make contact, and also for the cases marked *No fusion* and *No contact.*

A similar table might have been arranged for any of the other clones studied in this manner, but to take up so much space with tables would be both impracticable and unnecessary; therefore a single table has been prepared which gives the number of days and generations elapsing before the intraclonal reactions became negative (as indicated by shattering instead of fusing of the pro-

toplasms). This table (Table II.) shows a range of from 18 to 27 generations, and from 15 to 32 days, in the time of the first appearance of negative reactions between two lines of a clone, or an average of 22.25 generations and 21.58 days. After two lines had once become negative to each other no further cross-fusions took place between them unless certain modifications were made, the nature of which will be discussed later.

Table II.

Table Showing Date and Generation of First Exhibition of Shattering Reaction in Twelve Clones of Arcellæ. Both Lines of these Clones were Exposed to Similar Conditions.

Experiments Begun.	Clone.	Culture Medium used.	First Neg. Reaction Observed.			No. of Observations Made.
			No. of Generations.		Days Since Beginning.	
			1st. Line.	2d. Line.		
May 5..	*A*	Pond water	22	22	24	15
" 5..	*B*	" "	21	21	21	38
" 10..	*C*[1]	" "	—	—	—	3
" 5..	*D*[2]	" "	—	—	—	16
June 1..	*E*	25% hay infu.	23	27	32	25
" 1..	*F*	P.w. and H.i.	23	23	30	37
" 25..	*I*[3]	10% hay infu.	24	24	22	24
" 25..	*J*	" " "	18	18	15	39
" 25..	*K*	" " "	25	25	18	37
" 25..	*L*	" " "	20	20	17	30
" 25..	*O*	" " "	18	18	16	16
" 25..	*P*	" " "	19	20	18	21
Aug. 29..	*Q*	" " "	26	26	23	8
" 29..	*R*	" " "	25	24	23	18
		Average: 22.25 gen.			21.58 days.	

Before these cultures were discarded the culture medium, in which the organisms were living, was tested for its pH value by means of the Phenolsulphonephthalein indicator. In every case it was found to be neutral; *i.e.*, no detectable change had taken place because of the presence of the organisms.

Summary of Section I.

In summing up the results obtained from these experiments, the evidence indicates that physiological differences do develop

[1] Cross-fusion obtained on the 16th day. Cultures died before further observations were made.

[2] Cross-fusion obtained on the 10th day—cultures died.

[3] This set of experiments is given in extenso in Table I.

among the members of a clone, without necessarily being accompanied by corresponding morphological differences, and that these changes can be demonstrated by the cross-fusion method within relatively short periods of time—approximately 22 days. Since both lines of a given clone were subjected to the same external influences, the inference might be drawn that such protoplasmic changes are probably due to inherited variations and not to the action of environmental factors. However, later experiments show that this position is untenable.

II. Different Environments.

Having determined the time required for intra-clonal differentiation under similar environments, it was now possible to subject the two lines of a clone to different environments and observe the relative effects such treatment would have on their behavior towards each other's fragments. In carrying out these experiments such factors were employed as might occur in nature, viz: (*a*) 25 per cent hay infusion, (*b*) sucrose, (*c*) saline, (*d*) acetic acid, (*e*) sodium carbonate, (*f*) alcohol, (*g*) darkness, and (*h*) temperature. The organisms seemed to thrive best in 10 per cent hay infusion, and since this could be prepared by a given formula, it was used in every case (except one) as the standard culture medium: *i.e.*, one line of a clone was grown in it at room temperature in front of a window, while the other was altered as desired.

(*a*) *25 Per Cent Hay Infusion.*

An organism was isolated from a wild culture and placed in a concavity containing pond water. Upon dividing, the new cell thus formed was transferred to a receptacle containing 25 per cent hay infusion and designated as clone H line **h**-*hay*, while the individual remaining in the old shell was placed in a concavity containing fresh pond water and now became known as clone H line **h**. These two daughter-cells were then placed in the same moist chamber, so that the only difference in their environments was represented in the culture media. The usual tests for cross-fusion between the lines were made with the following results: on the second day after the beginning of the experiments +, 4th +, 6th +, 8th — o, 9th — — — — o — .[1]

[1] The significance of these symbols is as follows: + fusion occurred; — shatter-

A similar series of experiments were conducted between two diverging lines of clone W. In this case seven observations were made, extending through the eleventh generation, with the result that cross-fusion took place in every instance. Further observations were made impossible on account of death in one of the lines. In one case then, the time required for two related lines to exhibit the negative reaction was greatly reduced, while in the other death interfered with the determination of this point. If standing alone, further evidence would certainly be necessary before much emphasis could be placed on the significance of these observations; but, since the conclusions drawn, from the experiments dealing with different environments, were largely deducted from later observations, it was decided to include these as they stand without attempting to add more convincing proof.

(*b*) *10 Per Cent. Hay Infusion Containing 1 Per Cent. Sucrose.*

In these, and all succeeding experiments, the *standard* culture medium was 10 per cent. hay infusion. After the first division of the clonal parent, one of the daughter-cells was transferred to a concavity containing 10 per cent. hay infusion plus 1 per cent. sucrose, while the other was kept in the standard culture medium. In every other respect the two lines arising from these individuals were subjected to similar influences. In order to avoid possible contaminations, a different pipette was used in transferring members of these two lines to fresh culture media. The density of the medium was, of course, increased upon the addition of sugar, which resulted in the newly formed shells becoming smaller and smaller, so that it was considered advisable (perhaps necessary) to remove the osmotic influence after six or seven days; consequently, after having remained in the medium containing 1 per cent. sucrose for approximately that length of time, the organisms were put back into 10 per cent. hay infusion. From this time on both lines of a clone were living amidst the same surroundings. It was found that cross-fusion between the two lines continued for five or six days, then for a period of six or seven days the interactions were negative (even though the osmotic agent had

ing reaction; o contact made but neither fusion nor shattering followed. The number of symbols placed after a figure indicates the number of observations made on that day.

been removed) after which another short period of inter-fusion ensued. This is clearly shown in Table III.

TABLE III.

SHOWING INTERACTIONS OF RELATED LINES, **k** AND **k**–*su*. THE ENVIRONMENT WAS THE SAME FOR BOTH LINES, WITH THE EXCEPTION THAT FOR 7 DAYS LINE **k**–*su* WAS KEPT IN A 1 PER CENT. SUCROSE SOLUTION. THE EXPERIMENTS WERE BEGUN ON JUNE 27, AND DISCONTINUED ON JULY 22, 1922.

No. of Days after Beginning of Experiment.	Generation of Cell-body.	Generation of Fragment.	Horizontal Dimensions of Fragment in Micra.	Distance between Cell-body and Fragment in Micra.	Time in Minutes before Contact was Made.	Reaction after Contact.
2	**k**–*su*–3	**k**–2	9 x 34	75	10	Immediate fusion.
2	**k**–2	**k**–*su*–3	12 x 18	40	2	Fusion within 15″.
3	**k**–4	**k**–*su*–4	12 x 40	93	4	Fusion after 11′.
3	**k**–*su*–4	**k**–4	8 x 24	63	2	Fusion within 15″.
3	**k**–*su*–4	**k**–4	9 x 33	67	2.5	Fusion within 15″.
3	**k**–*su*–4	**k**–4	8 x 20	112	3	Fusion within 30″.
4	**k**–6	**k**–*su*–5	15 x 80	45	3	Immediate fusion.
4	**k**–6	**k**–*su*–5	8 x 35	60	3.5	Fusion within 30″.
4	**k**–*su*–5	**k**–6	8 x 45	0	0	Fusion after 2′.
6	**k**–8	**k**–*su*–6[1]	35 x 75	95	3	Shattering reaction.
8	**k**–9	**k**–*su*–7[2]	12 x 30	9	1	Shattering reaction.
9	**k**–10	**k**–*su*–9[3]	27 x 80	95	2	Shattering reaction.
9	**k**–*su*–9	**k**–10	12 x 15	55	–	No contact.
11	**k**–*su*–11	**k**–13	9 x 25	85	1	Shattering reaction.
11	**k**–13	**k**–*su*–11	8 x 14	32	0.5	No fusion.
13	**k**–16	**k**–*su*–14	24 x 26	26	2	No fusion.
13	**k**–*su*–14	**k**–16	10 x 45	13	1	Immediate fusion.
13	**k**–*su*–14	**k**–16	8 x 40	90	2	Immediate fusion.
13	**k**–16	**k**–*su*–14	9 x 32	63	1	Immediate fusion.
13	**k**–16	**k**–*su*–14	8 x 30	70	1	Immediate fusion.
13	**k**–*su*–14	**k**–16	25 x 30	40	2	Fusion within 1′.
13	**k**–16	**k**–*su*–14	10 x 56	85	2	Fusion within 1′.
14	**k**–17	**k**–*su*–15	12 x 30	140	4	No fusion.
15	**k**–19	**k**–*su*–16	10 x 20	115	2	Immediate fusion.
15	**k**–*su*–16	**k**–19	8 x 40	90	–	No contact.
20	**k**–*su*–21	**k**–24	25 x 60	37	4.5	Shattering reaction.
23	**k**–28	**k**–*su*–25	9 x 50	10	1	Shattering reaction.
23	**k**–*su*–25	**k**–28	8 x 10	33	1	Shattering reaction.
25	**k**–*su*–28	**k**–31	12 x 30	62	1	Shattering reaction.
25	**k**–31	**k**–*su*–28	8 x 25	35	1	Shattering reaction.

[1] Later specimen **k**–*su*–6 fused with its own fragment which had caused a shattering of specimen **k**–8's cytoplasm.

[2] The young individuals of line **k**–*su*, having been subjected to the influence of 1 per cent. sucrose for 7 days, were reduced in size to a diameter of 65 micra (the normal diameter being 100 micra). On this day line **k**–*su* was put back into 10 per cent. hay infusion, where it remained during the rest of the experiments.

[3] Later specimen **k**–*su*–9 fused with its own fragment after it had been abandoned by specimen **k**–10.

By referring to Table III. it will be seen that cross-fusion between the two lines persisted through generations **k**–*su*–5 and **k**–6, while two days later (**k**–*su*–6 and **k**–8) their potency to fuse with the other's fragments had ceased. On the 2d day after the first negative reaction was observed, line **k**–*su* was taken out of the sugar solution and placed in the same medium used for line **k**; nevertheless, the reactions between the two lines continued to be of the shattering type for 4 more days; *i.e.*, cross-fusion was not observed again until the fifth day after the two lines had been restored to a common environment (**k**–*su*–14 and **k**–16). For the next few days the inter-reactions were positive, but from generations **k**–*su*–21 and **k**–24 until the experiments were discontinued, shattering of the protoplasms took place in every instance. Compare the second shattering period with results shown in Table II.

An interesting point brought out by this study, is the fact that though the normal size was regained two days after the individuals of line **k**–*su* were removed from the sucrose solution, the inability to cross-fuse with fragments of specimens from line **k** persisted for five days. In other similar experiments the stability of this physiological variation as compared to the morphological difference in size was shown even more strikingly.

Three other series of experiments, in which 1 per cent. sucrose was used as the modifying factor, were conducted, and all of them gave results parallel to those shown in Table III. For instance, on June 7th the progenitor of clone M was isolated from a wild culture and upon dividing the next day, lines **m** and **m**–*su* were, started. Cross-fusion between these two lines was observed on the third day (generations **m**–4 and **m**–*su*–4), shattering reaction exhibited on the 6th (**m**–7 and **m**–*su*–6), line **m**–*su* put back in standard culture medium on the 7th, negative inter-actions persisted until the 15th (**m**–18 and **m**–*su*–15), then cross-fusions took place during the next five days, all of the interactions after this were of the shattering type. Twenty observations were made in this series of experiments.

Observations in clone J, lines **j** and **j**–*su* were begun on August 27th. Cross-fusion between line **j** and line **j**–*su* on the 4th day (**j**–4 and **j**–*su*–5), negative on the 6th. (**j**–6 and **j**–*su*–7), line **j**–*su* removed from 1 per cent. sucrose on 6th, negative inter-

actions persisted until the 15th (j–16 and j–*su*–16); this was followed by a brief period of cross-fusion, after which the interactions became permanently negative—number of observations in this case was 35.

A similar series of experiments between lines **n** and **n**–*su* of clone N were started on August 27th. In this case line **n**–*su* was removed from the sugar solution before the negative condition had been attained; consequently cross-fusion continued until the 15th day. From this time on all of the interactions were negative. Forty observations were made in this series of experiments.

(*c*) *10 Per Cent. Hay Infusion Containing N/10 Saline.*

Two series of experiments were conducted in which one line of a clone was placed in a culture medium of 10 per cent. hay infusion plus one tenth normal saline solution, while the other line was kept in the standard medium—the only difference between the environments of the two lines was that the culture medium used for one contained 0.09 per cent. salt. During the first three days the organisms in the saline solution seemed to grow and reproduce normally; after this, three days elapsed before another division occurred; then further growth ceased. In the meantime tests showed that cross-fusion between the two lines took place readily. After keeping the organisms in saline solution for eight days they were put back into standard culture medium. The interactions continued to be positive for two more days, then the experiments were discontinued.

The evidence obtained from these observations indicates that N/10 saline is an unfavorable environment for *A. polypora;* but regardless of this, no physiological change that can be detected by the cross-fusion method was thus produced.

(*d*) *10 Per Cent. Hay Infusion Containing Acetic Acid.*

Numerous attempts were made to culture the organisms in 10 per cent. hay infusion to which had been added various amounts of acetic acid, but after repeated efforts it was found impossible to keep the animals alive in solutions containing only 0.01 per cent. acetic acid. Other acids, or weaker concentrations of acetic acid, were not tried.

(*e*) *10 Per Cent Hay Infusion Containing 0.025 Per Cent Sodium Carbonate.*

By beginning with one per cent. sodium carbonate and each time decreasing the proportion, it was determined that *A. polypora* could be successfully grown in a medium containing 0.025 per cent. of this substance. Only one set of experiments was conducted, but the results obtained were very definite. On June 29th an individual was taken from clone K and set aside to become the progenitor of a new clone S. The next day it had divided, so one of the daughter-cells resulting from this division was placed in 10 per cent. hay infusion containing 0.025 per cent, sodium carbonate, while the other was kept in standard medium. The size, activity, and rate of reproduction of the organisms subjected to the influences of sodium carbonate (a weak base) seemed to be perfectly normal. Results of the reactions between line S and line S–*s.c.* are given by means of symbols (See footnote page 116 for meanings). On the second day after the experiments were begun the interactions were + +, 4th o o +, 6th line S–*s.c.* permanently transferred to standard medium, 8th − −, 9th + +, 11th + +, 14th − o, 19th −, 21st − − 23d − −. No further observations were made after this date. These results strongly suggest that, if one line of a clone be placed in 10 per cent. hay infusion and the other line in a similar medium to which 0.025 per cent. sodium carbonate has been added, the physiological constitutions of the members comprising the two lines will quickly become so differentiated that cross-fusion will not take place between individuals belonging to one line and fragments of specimens from the other. Such changes are shown to persist for some time after the causative agent has been removed.

(*f*) *10 Per Cent. Hay Infusion Containing 1 Per Cent. Alcohol.*

On August 28th specimens were taken from two clones, which had been under observation since June 25th, and with them two sub-clones, J and K, were started. These sub-clones were divided into two lines each; one of which was kept in standard medium, while the other was placed in 10 per cent. hay infusion plus 1 per cent. alcohol—the latter being designated sub-clone

J line **j**–*a*, and sub-clone K line **k**–*a* respectively. The organisms subjected to alcohol seemed to thrive quite well—the rate of fission, in both lines being slightly greater than that of those kept in standard medium. It was found, by testing the interactions of related specimens grown in these different environments, that the results were parallel in both cases; yet different from those obtained when substances other than alcohol were used for altering the environmental conditions. These observations were extended over a period of 65 days, representing 66 generations in sub-clone J and 63 in sub-clone K. The results are indicated by symbols (see footnote on page 116 for meanings). For sub-clone J, reactions between line **j** and line **j**–*a* were as follows: on the 3d day + o, 5th + o, 8th − − − −, 10th + +, 14th − − o −, 16th − o, 18th − o o, 21st + +, 23d + + +, 29th o o −, 33d + + +, 35th − −, 46th − o − −, 63d − −, 65th − o.

For cub-clone K, the reactions between line **k** and line **k**–*a* were as follows: on the 3d day o +, 5th + o, 7th +, 8th + + + +, 9th − − − −, 10th + + +, 11th + + +, 14th − −, 16th + o + o, 18th + +, 21st + + + + +, 23d + +, 28th − o o o, 30th + +, 35th + +, 46th o + +, 52d + +, 63d − −, 65th −.

As can be seen by glancing at these data, the interactions were variable and inconsistent. This suggests, perhaps, a fluctuating change of the protoplasm in contradistinction to the uniform conditions found to prevail in other experiments included in this section. No explanation of this is offered, since time did not permit an exhaustive investigation of the subject to be made; but it seems that one would hardly be justified in attributing the cause to a detrimental environment, for, as has already been dointed out, growth was not diminished by the presence of alcohol. Hegner ('19), found that *Arcella* dentata would grow and reproduce in a medium containing from 0.25 to 1 per cent. alcohol. However, the fission rate was retarded and irregularities in the shells of the offsprings were observed.

(g) *One Line of a Clone Kept in Darkness, the Other Exposed to Light.*

Having found that related specimens could readily be made negative to each other's protoplasmic fragments by adding various substances to the culture medium in which one line was grown, the following question arose: *Are the changes thus brought about due to the direct action of the added material, or to some other causes?* With this in mind, a series of experiments was begun in which the same culture medium was used for both lines of a clone, and in every other way they were treated alike, except one line was placed in the dark while the other was kept before a window. Thus, in one line the organisms were exposed to light during the day, while light was entirely excluded from those comprising the other, except for the short time required to transfer them to new depression slides (a period of about two minutes every other day). Four experiments of this type were conducted, involving clones G, T, U, and V. In three out of the four cases, the rate of reproduction was greater among the organisms kept in darkness than among their relatives which were exposed to light. However, it is possible that this was due to a greater abundance of certain forms of bacteria upon which the *Arcellæ* fed; for it was observed that the bacterial growth was usually very vigorous in the cultures which had been kept in the dark.

The results obtained in these experiments may be briefly given as follows: In clone G, line **g** and line **g**–*d* continued to inter-fuse with each other's protoplasm until the 15th day (16th generation); in clone T, the first negative reaction was observed on the 12th day (12th generation); in clone U, shattering reactions occurred between line **u** and line **u**–*d* on the 13th day (15th generation); in clone V, cross-fusion between line **v** and line **v**–*d* ceased on the 13th day (15th generation). After two related lines had once become negative, no further cross-fusions occurred.

In these experiments the average time required for two lines of a clone to become so differentiated as to shatter each other's detached fragments of protoplasm was 13.25 days, and 14.5 generations. While this is a longer period than was required for the shattering reaction to be exhibited when sucrose, for instance, was used as the differentiating factor, it is 8.33 days

(and 7.75 generations) shorter than was the case when no change was made in the environments. It is thus shown that the absence of light does affect, either directly or indirectly, the physiological constitution of *A. polypora*.

(*h*) *The Two Lines of a Clone Exposed to Different Temperatures.*

Specimens were exposed for twenty-four hours to temperatures of 36, 35, 30, 28, and 27 degrees centigrade, but in every case death occurred during the period of incubation. However, if incubated for only four hours, the organisms were able to resist a temperature of 36 degrees C. Tests were made to determine if such short exposures to heat would affect the protoplasm enough for it to be detected by the cross-fusion method. No changes of this sort could be revealed; regardless of whether the observations were made immediately after incubation, or twenty-four hours later.

Attempts to culture *A. polypora* at low temperatures proved to be more successful. Fifteen degrees centigrade was arbitrarily chosen as the low temperature environment, while room temperature (21 degrees C.) was used as the norm. A lower temperature was not selected because it was desirable that the organisms reproduce at a fairly rapid rate; though at 15 degrees C. the fission rate was reduced approximately one half.

On Nov. 7 two temperature experiments were started, involving Clone X line **x** and line **x**–*t*, and clone Y lines **y** and **y**–*t*. The same culture medium was used for both lines of a clone, and the lines which were not kept in the low temperature incubator were put in a darkened place to counteract any effect lack of light might have had on the organisms in the refrigerator. As usual, tests were made at frequent intervals to determine the interactions of two diverging lines. The results obtained are shown below by means of symbols.[1] In clone X—reactions between line **x** and line **x**–*t* on the 6th day + ¢ + o, 10th + ¢, 14th − − ¢ ¢, 16th o o, 17th ¢ − −, 19th − ¢ ¢.

In clone Y—reactions between line **y** and **y**–*t* on the 6th day ¢ − +, 8th + + +, 10th + +, 12th + + +, 14th + + +, 16th + o +, 17th o −, 19th − −.

[1] + signifies that fusion took place; − shattering reaction; o contact was made but neither fusion nor shattering followed; ¢ contact was not made. The number of symbols placed after a figure indicates the number of observations made on that day.

If the one shattering reaction observed on the 6th day of the experiment between the two lines of clone Y be disregarded, the above data will show that negative interactions first occurred on the 14th day in clone X, and on the 17th day in clone Y. Taking the higher figure 17 it is 4.58 earlier than the average time required when the environments in the two lines of a clone were similar. However, if we consider the number of generations, a greater difference is shown. On the day the first negative reaction was observed in clone X, line **x** had produced 15 generations and line **x**–*t* 8, which if added and the result divided by 2 yields 11.5 generations, while in clone Y, line **y** represented 18 generations and line **y**–*t* 10, or an average of 14 generations. Again, if we take the higher number it gives a decrease of 8.25 generations. It seems then, that if two lines of a given clone are subjected to different temperatures, they tend to diverge physiologically more rapidly than when kept at the same temperature.

Summary of Experiments Involving Different Environments.

As previously shown, when two lines arising from daughter-cells are subjected to similar environmental influences the property of fusing with each other's detached fragments of protoplasm ceases after approximately 21.58 days and 22.25 generations. By keeping one line in 10 per cent. hay infusion at room temperature in front of a window, such intra-clonal differentiation could be greatly hastened by altering (in only one respect) the other, as follows:

	Differentiation Exhibited.	
Factor imposed.	Days.	Generations.
25 per cent.[1] hay infusion used	8	9
1 per cent. sucrose added	6	7
N/10 saline added (unfavorable)		
0.01 per cent. acetic acid added (killed organisms)		
0.025 sodium carbonate added	7	7
1 per cent. alcohol added (fluctuated between + and −)		
Kept in darkness	13.25	14.5
Kept at 15 degrees C.[2]	16.5	12.25

[1] The other line kept in pond water instead of 10 per cent. hay infusion.

[2] The other line kept in darkened place to counteract darkness of refrigerator.

III. Identical Environments.

When the experiments involving similar environments had been completed, and before the experiments described in section II had been performed, it *seemed* that the inability of an organism to fuse with a protoplasmic fragment which had been severed from a related individual was due to inherited physiological variations. However, it was found, as shown in section II, that by employing different environments the negative state was reached in a much shorter time than was the case when the conditions were unaltered. These results indicate either (1) that the different environments have brought about heritable physiological changes which influence the character of the reactions, or (2) that the different environments have brought about temporary physiological changes which do not persist after the organisms are returned to identical surroundings. In other words: the so-called "similar" environments may have, in reality, been different. As Jennings ('16) pointed out, and Hargitt and Fray ('17) and Phillips ('22) have shown by feeding *Paramecia* on pure cultures of bacteria, food is an important factor in bringing about modifications. There seems to be no doubt that certain forms of bacteria prevail in one concavity while in an adjoining concavity the flora may be entirely different. *The Arcellæ* in hay infusion cultures subsist principally on the bacteria by which they are surrounded; therefore, one should not be surprised if physiological differences are exhibited between organisms kept in different containers.

Several methods might be employed in determining whether or not the bacterial flora available to the organisms of two contrasting lines are similar:

1. By making cultures of the medium in which they are living, and determining the kinds and relative proportions of bacteria present through the use of differential culture media. However, since little is known about hay infusion bacteria, this would be a difficult undertaking.

2. By isolating a pure strain of suitable bacteria and feeding them to the organisms under aseptic conditions.

3. (*a*) By frequently transferring small quantities of the medium from one concavity to the other, and (*b*) by placing both

organisms in the same concavity and removing the new individuals as they appear.

Methods 1 and 2 were not undertaken, but both (*a*) and (*b*) of method 3 were utilized.

Four clones, J, K, Q, and R were started and each of these was divided into two lines upon the first division. Individuals from these lines were transferred to new concavities every other day, and upon each transferral of the organisms a quantity of the culture medium from the old concavity in which one line had been growing was placed in the new concavity in which a specimen from the other line was to be confined, and *vice versa*. Thus, each time an organism was placed into fresh culture medium it brought with it some of the bacteria from the old culture in which it had been living; but in addition to this, bacteria from the old culture in which its sister line had been growing were also introduced, so that any difference in the flora existing in the two old concavities were distributed to the new concavities alike. Tests were made between representatives of the two lines as had been done in former experiments, and it was found that cross-fusion did not cease after twenty or twenty-five generations, but persisted as long as the experiments were continued; or in clone J until the 42d generation, clone K until the 47th, clone Q until the 65th, and clone R until the 58th. After obtaining cross-fusion in clones J and K for 42 and 47 generations respectively, Petri dish cultures were made of the two lines in each clone by putting four individuals in a Petri dish containing 25 c.c. of 10 per cent. hay infusion. These were placed before a window where they remained undisturbed from July 31 until August 25.

Another series of experiments was begun in which attempts were made to bring related specimens, that had been reacting negatively towards each other's fragments, to a state in which cross-fusion would take place between them, by frequently exchanging small quantities of culture medium between the concavities in which they were living. Three variations of this system were employed: (*a*) by cross-transferring a small quantity of culture medium from the old receptacle of one line to the new container of the other each time the organisms were conveyed to fresh culture medium—thus bringing their environments

to a common state; (*b*) by transferring medium only from one line to the other—thus making the conditions in the latter like those in the former; (*c*) the environments of both lines were changed through the use of a different culture medium; these were then brought to a common identity by exchanging small quantities of culture medium between them.

Members from the Petri dish cultures of clones J and K were used for this purpose. When tested on August 25 it was found that the two lines of these clones reacted negatively towards each other's fragments. By estimating the number of individuals in each Petri dish, and knowing the number of days the cultures had been running and their usual rate of reproduction, a fairly accurate determination could be made of the number of generations represented in each line. In every case the number used was slightly under the estimated number. By following this procedure the figure obtained on August 25 were: for clone J, line **j**–66 generations and line **jd**–60; for clone K, line **k**–70 and line **kd**–68. The same day three types of experiments were begun in which both of these clones were used.

(*a*) Two small drops of culture media were interchanged between the old receptacles containing specimens from one line and the new concavities into which a specimen from the other line was to be placed each time the organisms were conveyed to fresh cultures (every two days). At intervals of two or three days observations were made of the reactions between individuals from the two lines. In clone J the interactions of lines **j** and **jd** continued to be of the shattering type for 18 days (until September 12), when cross-fusion was exhibited (line **j**–84 and line **jd**–80). From this time until the experiments were discontinued on November 1 (**j**–124 and **jd**–119), individuals from one line would readily fuse with fragments that had been severed from members of a sister line. In clone K the interactions between lines **k** and **kd** were of the shattering type for 20 days (until September 14). From this time on, cross-fusion between the two lines was exhibited, though several shattering reactions were also observed. The experiment was discontinued on November 1, at which time line **k** had undergone 135, and line **kd** 132 divisions since clone K was started on June 25.

(*b*) In the following experiments attempts were made to bring

lines **jd** and **kd** to the conditions of lines **j** and **k** respectively, by conveying some of the culture media in which the latter had been living to the concavities containing the former. Under this treatment individuals from lines **j** and **jd** reacted negatively towards each other's fragments during the succeeding fifteen generations (until September 9). After this cross-fusions were the usual occurrences, though occasional shattering reactions were encountered. When the experiment was discontinued on November 1 line **j** represented 129 generations and line **jd** 120 generations since the beginning of clone J. The reactions between lines **k** and **kd** continued to be of the shattering type until September 16 (for 24 generations). From then until the experiment was discontinued on November 1 (line **k**–135 and line **kd**–133) most of the interactions were positive.

(*c*) In these experiments specimens from clone J, lines **j** and **jd** and clone K, lines **k** and **kd** were taken from the Petri dish cultures and placed in concavities containing 10 per cent. hay infusion plus 1 per cent. sucrose; but after remaining in this solution for six days, the organisms had decreased so much in size that they were put back in standard medium. Every other day small quantities of culture media were exchanged between the receptacles in which sister lines were kept. The organisms subjected to 1 per cent. sucrose not only decreased in size, but their physiological states were so altered that they no longer fused with fragments from closely related individuals which had been kept in standard culture medium—see section II, (*b*). Thus, it is evident that an environment different from what existed before had been established.

Upon testing by the usual method, it was found that the shattering type of reactions persisted between specimens of line **j** and line **jd** for 18 days and 19 generations, but from then on, cross-fusion was exhibited. The experiment was discontinued on Nov, 2d, at which time line **j** represented 125 generations and line **jd** 122. In clone K shattering reactions were observed between line **k** and line **kd** until the 23d day and 17th generation after the experiment was started. Later observations yielded a much larger proportion of shattering reactions than were encountered in any of the other experiments, but a number of cross-fusions did take place between the two lines. When the

experiment was stopped on Nov. 1st. line **k** had undergone 119 divisions and line **kd** 122.

The results obtained in the above experiments indicate that the changes produced in the protoplasm of related individuals reproducing vegetatively, are at least greatly influenced by the environment—in fact to such an extent that two lines which have been negative to each other's protoplasmic fragments for several weeks can be brought back to a common condition in which cross-fusion will take place between them within approximately 20 days. However convincing this evidence seems, it was considered advisable to obtain further proof. To that end, a series of experiments was planned which would extend the observations over a longer period, and at the same time remove any question as to the identity of the environments in which the two lines of a clone were existing. It is a matter of common knowledge, that when *Arcella* divides the newly formed shell is lighter in color than the old shell, and that this difference in shell coloration continues for some time after division takes place. Therefore an individual possessing a dark shell was taken from each of the two diverging lines of a given clone and placed in the same concavity. Then daily examinations were made, and when two specimens with light-colored shells appeared, they were removed and tested for inter-protoplasmic reactions; while the two dark-shelled organisms were transferred to fresh culture medium. It might be said in this connection, that the morphological differences between specimens from the two lines, were usually also great enough to enable a careful observer to distinguish one from the other.

On November 1, 1922, four experiments of this type were started, involving clone J, line **j**–124 and line **jd**–119; clone K, line **k**–135 and line **kd**–132; clone Q, line **q**–65 and line **qd**–65; and clone R, line **r**–58 and line **rd**–58. The two lines of each of these clones had previously been brought to a common condition by exchanging small quantities of culture media from one to the other, as described elsewhere; so that individuals from one line would fuse with fragments of members of the other at the beginning of these experiments. So then, it was to be expected that the immediate progeny of two such organisms would also exhibit the cross-fusion phenomenon.

Individuals from the two lines of these clones were confined in the same concavities for ten days. During this time frequent tests showed cross-fusion in every instance: consequently on November 10 the related lines were separated again, by placing the individuals in different Petri dishes containing 25 cc. of 10 per cent. hay infusion. At intervals of two weeks the number of individuals in the Petri dish cultures were estimated, and the approximate number of generations represented determined. Then fresh Petri dish cultures were made, and thus the lines were perpetuated until January 5, 1923.[1] On January 5 tests were made for cross-fusion between the two lines of these clones, with the result that all of them gave the shattering reaction. Then an individual from line **j** was put in a concavity with an individual from line **jd**. In a like manner the two lines of the other clones were brought together. During the next five days the inter-protoplasmic reactions were universally negative, while on the sixth day they were all positive. Cross-fusion between the related lines continued until the experiments were stopped on January 15. At that time clone J had been observed for 188 generations, clone K for 200, clone Q for 126, and clone R for 122.

These results present a strong argument in favor of environments as the principal modifying agent; but the skeptic might say that perhaps the two lines were confused while being kept in the same container, and what seemed to be cross-fusion between very distantly related individuals was really positive interactions of very closely related specimens. That such was the case is very improbable; for in the first place, especial care was taken to avoid that error, and secondly, the results obtained were too uniform. The same mistake would hardly have been made in every experiment at the same time. However, the most convincing evidence obtained was made possible by the sudden appearance of a double form in a Petri dish culture of clone Q, line **qd**. This abnormal specimen was discovered on November 21, 1922, and seems to have appeared because of the failure of the shells to completely separate at the time of fission. It is

[1] An objection might be raised to mass cultures on the grounds that conjugation might take place between some of the organisms, thus disturbing the germinal constituency of the individuals concerned. This possibility was taken into consideration and guarded against, and it can be said with assurance that conjugation did not take place among the specimens used in this investigation.

shown contrasted with a normal individual in Fig. 4. (See Reynolds '23.)

As can readily be seen, the morphological differences between this abnormal specimen and a normal individual were quite marked; consequently they could be kept in the same concavity without any danger of confusion. The abnormal form could be reared, and it was found to breed true to type. By January 12 34 generations had been obtained from the abnormal *Arcella*, while the normal individuals of the line from which it sprung had given rise to 37 generations. During this time, a period of 52 days, the normal and abnormal organisms had been kept in different receptacles, and no exchange of medium between them had been made. In accordance with other findings, therefore, they should have reacted negatively towards each other's fragments of protoplasm—even though both had possessed the same morphological characteristics. In testing this out on January 12 it was found that they exhibited a violent shattering reaction. On the same day an abnormal specimen **Ab**–34 was put in the same concavity with a normal organism **qd**–123, both of which belonged to the same line (**qd**) of clone Q. By making daily tests[1] it was found that the interactions continued to be negative for three days. From the fourth day on, cross-fusion took place between them readily.

Another experiment involving the abnormal form was also started on January 12. In this case a normal individual **j**–185, which was descended from a specimen taken from a wild culture on June 26, 1922,[2] was placed in a concavity with an abnormal organism **Ab**–34, after it had been demonstrated experimentally that one would not fuse with fragments of the other's protoplasm. Then the usual procedure of daily testing the interactions between the progeny of these two organisms was carefully practiced. During the first five days the involved protoplasms were shat-

[1] In this and the following experiment all amputations, and subsequent observations of protoplasmic reactions, were made without removing the organisms from their containers.

[2] While the two organisms used in this experiment belonged to the same species, and probably at some time in the past had a common ancestor (since their progenitors were isolated from the same wild culture); their relationship was certainly remote, for the two clones to which they belonged had been under observation for nearly seven months.

tered to pieces upon making contact, after which the animal would move off, leaving the remnants of its own and the fragment's protoplasm behind. On the 6th, 7th, and 8th days contact was followed by a slight shattering of protoplasm, and later some or all of the remnants were appropriated by the reacting organism. On the 9th, 10th, 11th, and 12th days of coexistence one organism would fuse with a fragment of the other practically, if not quite, as readily as with one of its own fragments. No further observations were made after the twelfth day.

Summary of Experiments Involving Identical Environments.

By continually interchanging small quantities of media from old cultures of one line of a given clone to new cultures of the other line, cross-fusion between the two lines can be prolonged for sixty-five generations (perhaps indefinitely?). After two lines of a clone have ceased to fuse with each other's fragments, the property of cross-fusion can be restored by: (*a*) frequently exchanging small quantities of media between the cultures—thus bringing them to a common state; (*b*) frequently transferring small quantities of medium from only one culture to the other—thus bringing one line to the condition of the other; (*c*) creating a different environment in both lines by adding 1 per cent. sucrose to the culture medium and then exchanging media between them frequently. Quicker and more satisfactory results can be obtained, if members of the two lines to be tested are cultivated in the same container. By this method, two lines of a clone that have been negative to each other's protoplasm for months, can be brought back to a state of cross-fusibility—even though they are removed from the clonal parent by as many as 200 generations. A normal specimen was put in the same concavity with an abnormal relative. Though reacting negatively in the beginning, they readily fused with each other's fragments of protoplasm after living together for four days. An unrelated (?) normal specimen was placed in the same container with an abnormal *Arcella*. In this case the shattering reaction was exhibited until the ninth day of coexistence; from then on, fusion occurred readily between one organism and pseudopodial fragments of the other.

Discussion.

Before entering into a discussion of the results obtained in these investigations, it is perhaps advisable to show that the method employed is sound, and that it offers some advantages over the methods which have been utilized by others. As stated in the introduction, this is the first time that the interactions of protoplasmic masses have been used in studying the subject of Heredity and Environment. It was not until the recent appearance of the first article of this series that anything was published concerning the fact that certain of the shelled rhizopods are capable of reappropriating, by fusion, fragments of protoplasm which have recently been detached from their cell-bodies. In this paper it is clearly pointed out that "the severed fragments are not recovered as food, but enter again immediately into the protoplasmic structure of the cell-body." Later work has strengthened the evidence for such a contention. In fact, the findings in these experiments with *A. polypora* substantiate all of the conclusions drawn from the work on *Difflugia*, except paragraphs 3 and 5 of the general summary.

In regard to paragraph 3: In *Arcella*, fusion has been observed to occur at the ends of pseudopods, as well as along an extended mid-region. This difference in behavior of the two genera may be due to the difference in the nature of their pseudopodial formation. As to paragraph 5: In six cases out of approximately 1,000 two enucleated fragments have been observed to fuse with each other; though in such instances they were severed from the same cell-body at the same time, and were almost in contact in the beginning.

In addition to the data on protoplasmic fusion given in Article No. 1: the "shattering" reaction is described in the introduction of the present paper. This is the kind of reaction usually observed when an organism made contact with a fragment severed from a distantly related individual. It was found that in such cases there was a mutual attraction between the organism and the fragment—even though their protoplasms were shattered upon making contact. If the fragment came from a closely related specimen fusion occurred. When a fragment of protoplasm was severed from a different genus (*Difflugia*) and placed

near an *Arcella* the two bodies did not attract eachother. This was done on ten different occasions; each time the fragment was placed within 100 micra of the organism, but neither body showed any response to the other's presence. Chance wanderings should have brought the two masses together in a certain percentage of cases. This did happen on two occasions out of the ten tried, but nothing resulted from the contact. In both instances the animal moved on as if the fragment had been merely a mechanical obstruction.

Another observation which is probably worthy of mention in this connection, is that under ordinary circumstances two *Arcellæ*, which would react negatively towards each other's fragments, may come in contact with each other's pseudopods without causing any disturbance; but when a protoplasmic fragment of one of these organisms is in their midst, it was frequently observed that their pseudopods would be shattered upon making contact.

With these various facts established, it seems evident that the inability of an organism to fuse with a pseudopodial fragment is due to a difference in the physiological constitution of the protoplasm involved. It is generally considered that morphological differences are merely physical expressions of physiological changes—a kind of cause and effect relationship. If this be true, then physiological tests offer finer distinctions between minute variations than can be perceived in the structure of organisms. Furthermore, as shown by the experiments in which one line of a clone was placed in a culture medium containing 1 per cent. sucrose, physiological changes may persist for a long time after morphological differences have disappeared.

Most investigators in the past have, of necessity, studied variations among protozoa by making use of structural differences. While these are undoubtedly of great importance, they do not offer as delicate a method of determining variations, or as sure a means of detecting them. Anyone who has observed a "shattering" reaction can but be impressed with the fact that a marked difference exists between the reacting masses—a few generations back they were parts of the same individual; they have diverged by means of binary fission until they not only fail to unite when brought together, but are shattered into many pieces. Why does this happen? We can postulate many possible

explanations. It seems most reasonable to think of it as being due to a difference in molecular structure, or ionization; at least the sudden violent shattering is suggestive of an electrical phenomenon. The inability of one organism to fuse with a protoplasmic fragment of another, does not imply that the two organisms concerned differ structurally, nor does cross-fusion indicate that no morphological differences exist. In either case the animals involved may or may not be identical in size, structure and rate of fission.

Maupas ('88, '89), in studying the effect of temperature on the fission rate of infusoria, arrived at the conclusion that under given conditions the fission rate remains the same, and that inherited variations do not exist. Regardless of the important bearing this statement has on the theory of evolution, there was but very little interest exhibited until some time after the publication of Johannsen's ('03) results, in which he enunciated his concept of genotypes. Since then workers in various fields have investigated the subject of Heredity and Environment. Like Maupas, most of them have obtained evidence which seems to emphasize the "fixity" of species. Among these might be mentioned: Johannsen ('09, '11) working with beans, Hanel ('08) and Lashley ('15) with *Hydra*, Jennings ('08, '09, '10) with infusoria, Ackert ('16) with *Paramecium*, and Jollos ('21) with infusoria. However, recently Barber ('07) working with bacteria, Jennings ('16) with *Difflugia*, Hegner ('19) with *Arcella*, Root ('18) with *Centropyxis*, Middleton ('15, '18) with *Stylonychia*, Erdmann ('20) with *Paramecium*, and others, have apparently been able to demonstrate that variations do take place among organisms reproducing asexually—and as Erdmann ('20) says: "The rigid conception of the genotype does not hold true for protozoa."

To enter a field filled with so much conflicting evidence, armed with a new method, was an especially attractive undertaking. The experiments were planned with the idea of duplicating, so far as possible, the various conditions to which protozoa have been subjected by others. The only new factors employed were the method of determining variations which might arise, and a little different way of preparing the culture medium.

The results obtained seem to combine the two diverse opin-

ions, and thus answer some of the hitherto perplexing questions. In the first place, they show that both physiological and morphological variations do appear among protozoa reproducing vegetatively, and that such variations can be hastened by altering the environments; and secondly, that these changes are not due to inherited variations which persist for very long periods of time, for they could be reversed within a few days. Unquestionably, certain heritable variations often arise in cultures, and seem to persist for some time afterwards; but, in our experiments at least, if the organisms in which these changes are manifested are left in the same surroundings, undisturbed, they tend to revert to the normal type, while if placed in a different environment where selection would be affective, or if artificial selection is practiced, the aberrant characteristics may be continued, or even emphasized.

In a similar manner the physiological status of organisms is subject to modifications. Adjustments are constantly being made as a result of the ever changing surroundings. The question seems to resolve itself into one of environmental factors, of which food is by no means the least important. The only difficult factor to control in these experiments was the bacterial growth in the culture media. In fact, the rapid differentiation observed when unlike culture media were used, was probably due to a change in the character of the bacterial flora produced by the action of the environment; for if the action had been direct, in some cases at least, physiological differences should have occurred within a few hours after the organisms were exposed to it.

Summary and Conclusions.

1. Like *Difflugia*, *Arcella polypora* will, under favorable conditions, reappropriate its detached fragments of protoplasm by fusion.

2. *A. polypora* is not attracted by a pseudopodial fragment of *Difflugia*. If accidental contact is made the fragment is treated as a mechanical obstruction.

3. Fusion will take place between one individual and a protoplasmic fragment of a closely related specimen.

4. Two distantly related individuals, which have not been kept in the same receptacle, will be attracted by each other's

severed pseudopods, but upon making contact the involved protoplasms will be shattered into bead-like masses.

5. When two lines of a clone are kept under similar environments, cross-fusions between them cease after approximately 22 days; while if the two diverging lines are subjected to different environments, the time required for cross-fusions to cease is greatly shortened (varying from 6 to 16.5 days depending on the factor employed).

6. When small quantities of culture media are frequently exchanged between the concavities in which members of two diverging lines are confined, cross-fusions between them apparently continue indefinitely

7. After two related specimens have become negative to each other's protoplasm, cross-fusions between their progeny may be induced by (*a*) exchanging small quantities of culture media between the receptacles in which they are kept (time required was 20 days), or (*b*) by placing both of them in the same concavity (time required was 6 days).

8. Morphological differences are not necessarily associated with different physiological states, as evidenced by the fact that abnormal specimens could be induced to fuse with fragments of normal individuals—and *vice versa*.

9. Physiological changes do occur among the descendants of a single *A. polypora* reproducing vegetatively, but such differences are probably due to environmental influences rather than to hereditary variations.

BIBLIOGRAPHY.

Ackert, J.

'16 On the Effect of Selection in *Paramecium*. Genetics, Vol. 1, 387–405.

Barber, M. A.

'07 On Heredity in Certain Microörganisms. Kansas University Science, Bul. 4, 3–47.

Calkins, G. N.

'11 Regeneration and Cell Division in *Uronychia*. Jour. Exp. Zoöl., Vol. 10, 95–116.

Calkins, G. N., and Gregory, L. H.

'13 Variations in the Progeny of a Single Ex-conjugant of *Paramecium caudatum*. Jour. Exp. Zoöl., Vol. 15, 467–525.

Castle, W. E.

'14 Pure Lines and Selection. Jour. Heredity, Vol. 5, 93–97.

Erdmann, Rh.

'20 Endomixis and Size Variations in Pure-bred Lines of *Paramecium aurelia*. Arch. f. Entwicklungsmech., Bd. 46, 85–146.

Hanel, Elise.

'08 Vererbung bei ungeschlechtlicher Fortpflanzung von *Hydra grisea*. Jenaische Zeitschr., Bd. 43, 321–372.

Hargitt, G. T. and Fray, W. W.

'17 The Growth of *Paramecium* in pure Cultures of Bacteria. Jour. Exp. Zoöl., Vol. 22, 421–455.

Hegner, R. W.

'19 Heredity, Variation and the Appearance of Diversities during the Vegetative Reproduction of *Arcella dentata*. Genetics, Vol. 4, 95–150.

'19a The Effects of Environmental Factors upon the Heritable Characteristics of *Arcella dentata* and *A. Polypora*. Jour. Exp. Zoöl., Vol. 29: 427–441.

'20 The Relations between Nuclear Number, Chromatin Mass, Cytoplasmic Mass, and Shell Characteristics in Four Species of the Genus *Arcella*. Jour. Exp. Zoöl., Vol. 30, 1–95.

Jennings, H. S.

'08 Heredity, Variation and Evolution in Protozoa. II. Heredity and Variation in size and form in *Paramecium*, with Studies of Growth, Environmental Action and Selection. Proc. Amer. Philos. Soc., Vol. 47, 393–546.

'16 Heredity, Variation and the Results of Selection in the Uniparental Reproduction of *Difflugia corona*. Genetics, Vol. 1, 407–534.

Johannsen, W.

'03 Ueber Erblichkeit in Populationen und in reinen Linien. v + 68 pp. Jena: Gustav Fischer.

Jollos, Victor.

'21 Experimentelle Protistenstudien. Arch. f. Protistenk., Bd. 43, 1–222.

Kepner, W. A., and Reynolds, B. D.

'23 Reactions of Cell-bodies and Pseudopodial Fragments of *Difflugia*. Biol. Bul., Vol. 44, 22–47.

Lashley, K. S.

'15 Inheritance in the Asexual Reproduction of *Hydra*. Jour. Exp. Zoöl., Vol. 19, 157–210.

Leidy, Joseph.

'79 Fresh-water Rhizopods of North America. Report U. S. Geol. Survey of the Territories, Vol. XII, xi + 324 pp. Washington: Government Printing Office.

Middleton, A. B.

'15 Heritable Variations and the Result of Selection in the Fission rate of *Stylonychia pustulata*. Jour. Exp. Zoöl., Vol. 19, 451–502.

'18 Heritable effects of Temperature Differences in the Fission rate of *Stylonychia pustulata*. Genetics, Vol. 3, 535–572.

Penard, E.

'02 Faune Rhizopodique du Bassin du Léman. 714 pp. Geneva: W. Kundig et fils.

Phillips, R. L.

'22 The Growth of *Paramecium* in Infusions of Known Bacterial Content. Jour. Exp. Zoöl., Vol. 36, 135–183.

Popoff, M.

'08 Experimentelle Zellstudien I. Arch. f. Zellforsch., Bd. 1, 245–379.

'09 II., Bd. 3, 125–178.

Reynolds, Bruce D.

'23 Inheritance of Double Characteristics in *Arcella Polypora* Penard; Genetics. Vol. 8, 477–493.

Root, F. M.

'18 Inheritance in the Asexual Reproduction of *Centropyxis aculeata*. Genetics, Vol. 3, 174–206.

Stout, A. B.

'15 The Establishment of Varieties in *Coleus* by the *Selection of Somatic Variations*. *Carnegie Institution of Washington*, Pub. No. 218: + 80 pp.

Whitney, D. D.

'16 The Transformation of *Brachionus pala* into *B. amphiceros* by Sodium Silicate. BIOL. BUL., Vol. 31, 113–120.

EXPLANATION OF PLATE I.

FIG. 1. Camera lucida sketches illustrating shattering reaction. *A*, *Arcella polypora* approaching a fragment from another individual of the same species, fragment shown below. × *170*. *B*, The same bodies just after making contact. *C*, A portion of *B* enlarged.

FIG. 2. Diagram of vertical section illustrating shape of shell and position of pseudopods.

FIG. 4. An abnormal *Arcella* which appeared suddenly in a hay infusion culture of clone Q, line **qd**. *A*, Lateral view; *B*, Ventral aspect, showing ellipsoidal mouth; *C*, normal individual of line from which the abnormal specimen was obtained. × *170—camera lucida*.

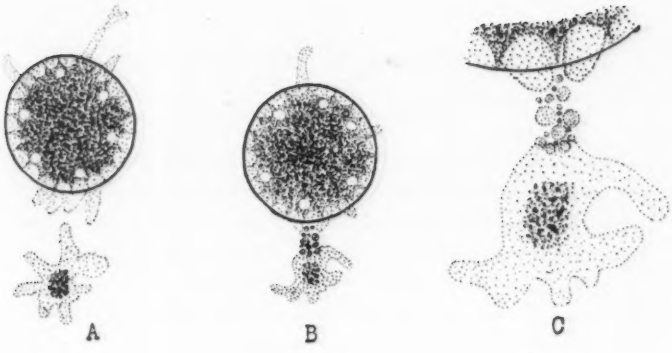

Fig. 1.

Fig. 2.

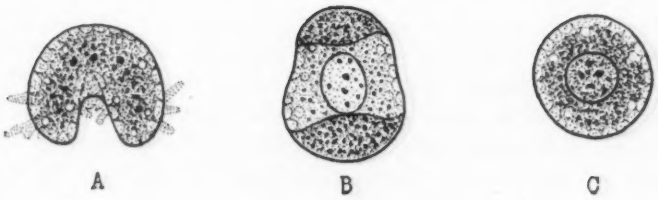

Fig. 4.

BRUCE D. REYNOLDS.

THE ATTACHMENT OF OYSTER LARVÆ.

THURLOW C. NELSON.[1]

Knowledge of the life history and ecology of the American oyster, *Ostrea virginica* Gmelin, is more extensive than in the case of any other species of lamellibranch. The researches of numerous investigators, notably of Brooks, J. Nelson, and Stafford, give us an almost unbroken history of this mollusc from the egg to the adult. As in most marine bivalves, the eggs of the oyster are shed into the water where fertilization occurs. Subsequent development is rapid, the larva forming a bivalve shell within two days or less. Then there follows a pelagic period of approximately two weeks during which the larva swims about with the aid of the velum, and increases considerably in size. At the close of the free-swimming period the larva settles upon some solid object and remains firmly attached at that point for the remainder of its life. Up to the time of attachment the history of most other marine lamellibranchs is similar to that of the oyster, but once the free-swimming period is ended we find a variety of behavior; the larvæ of *Pecten*, *Modiolus*, and *Mytilus* attach to seaweeds or to other objects, the larvæ of the Teredinidæ and of the Pholads bore within a solid substratum, while those of *Venus* and *Mya* burrow into the bottom.

Concerning the actual attachment of the oyster larva at the close of its pelagic life we have little information, the nature of the process having been deduced in the main from a study of the stages just preceding and just following fixation. Ryder, '82, Huxley, '83, Jackson, '90 and others, including the writer (Nelson, '21 A), believed that the secreting border of the mantle was used to cement the larva fast to the substratum. Stafford, '13, on the other hand, found in his youngest oyster "spat" that the left valve bore a layer about five times as thick as the shell itself, composed apparently of a different material. This thick layer

[1] From the Zoölogical Laboratory of Rutgers College and the Department of Biology, New Jersey Agricultural Experiment Station. Paper No. 117 of the Journal Series, New Jersey Agricultural Experiment Stations, Department of Biology. This paper will appear in Rutgers College Studies Vol. 2.

covers more than one half of the outer surface of the left valve, extending from close to the anterior ventral edge almost to the umbo, but falling short posteriorly. From his study of the byssus gland in the base of the foot of the larval oyster, Stafford concluded that this organ must furnish the substance used to attach the larva, since the large amount of this material and its position far under the left valve preclude the use of the mantle for this purpose.

It has been my good fortune to observe the actual attachment of oyster larvæ under semi-natural conditions, and thus to be able to fill the gap in the information about this process. Large numbers of full grown oyster larvae were found at 8 P.M., July 23, in the water surrounding our laboratory houseboat on Barnegat Bay. A glass plate measuring 3 x 4 inches was lowered vertically into the water and removed half an hour later, and immediately suspended horizontally in a dish of sea water under the binocular.[1]

Six oyster larvae were moving about over the glass, holding on by means of the very active and highly adhesive ciliated foot. Some were on the upper, others on the lower side of the glass, thus permitting observation of the attachment process both from above and from below. With the velum withdrawn, and slowly

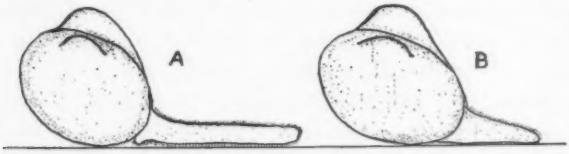

FIG. 1. Illustration of the method of creeping of the oyster larva over a glass surface. *A*, larva with foot fully extended; *B*, larva with foot contracted and body pulled forward. Larvæ shown from the right side.

rolling the shell from side to side, the larvæ extended the foot to a distance of about 0.25 mm., attached the distal end and then a contraction of the foot pulled the body forward. The "heel" of the foot next being attached, the body was swung part way around; the tip of the foot was again extended in the new direction and the process repeated (Fig. 1). When first observed at

[1] Jackson, '88, J. Nelson, '08, and Stafford, '10, used this method of obtaining oyster spat; but no one of these authors records having witnessed the actual attachment of the larva.

8.35 P.M. the larvæ were describing circles a little over 1 mm. in diameter. As they continued moving around the radius of the circles was gradually diminished, until at 8.46 the first larva came to rest With the foot extended to about one half its full length, its distal end flattened in contact with the glass, the larva swung the body until the foot occupied the median position, with the left valve against the glass and inclined to it at about 30 degrees (Fig. 2). In this position the ventral edge of the left valve almost touches the substratum. The ventral border of the mantle was extended until it came in contact with the glass where it remained for 2 minutes, and then was withdrawn. The foot, with its cilia beating very feebly, was then slowly drawn in, and 11 minutes after the larva ceased circling the foot had entirely disappeared between the valves, and fixation was accomplished. The method of attachment of the larvæ on the upper and on the lower side of the glass was the same; it illustrates, in the case of those larvæ attaching from below, the power of the foot in holding the left valve firmly against the substratum while fixation is being effected.

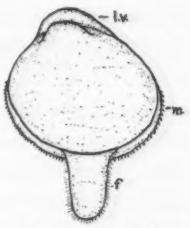

FIG. 2. The position taken by an oyster larva during fixation, shown from right side. *f*, foot; *l. v.*, umbo of left valve; *m*, mantle.

The great importance of the foot to the full-grown oyster larva was recognized by Stafford '10, who described its creeping movements. The manner in which the larva cements itself fast was deduced by him ('13, p. 65) solely from histological evidence. He concluded that the mantle could not furnish material in sufficient quantity nor rapidly enough to be of use as the organ of fixation. As he says: "The usefulness of the foot as an organ of locomotion, as a clinging organ, as an organ of fixation, had appealed to me for some time but I had no direct evidence to support the view that it was really the organ of final attachment."

Stafford found that a large part of the base of the foot of a full-grown oyster larva is occupied by the byssus gland which consists of three lobes communicating with a duct which opens

to the outside at the heel of the foot. The cells of this gland are gorged with a transparent secretion which is not found in the much shrunken gland which characterizes the larva immediately after attachment. He believed that fixation was accomplished by the larva extending the foot until the heel came to a position near the upper anterior edge of the valve, and that the byssus gland then discharged its secretion, which, flowing between the left valve and the substratum, soon hardened and held the larva fast. Stafford believed that the mantle played no part in the fixation process.

My own observations show that Stafford was right in considering the foot as the organ of final attachment. It is not thrust forward, however, as he held, but is brought to the median position. The extrusion of the mantle for a short period evidently aids in the quick and economical distribution of the cementing fluid as it is poured out of the byssus gland at the ventral edge of the left valve. This secretion hardens in less than 10 minutes. In Fig. 3 are shown a number of newly attached larvæ

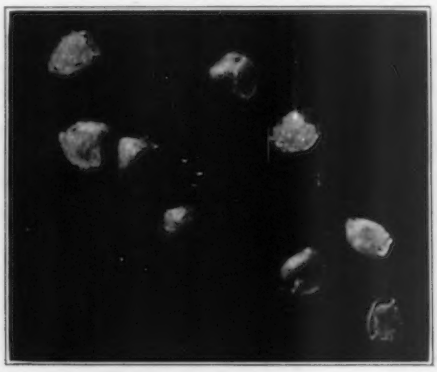

FIG. 3. Oyster larvæ removed just after attachment and photographed from the left side to show the area covered by the cementing substance from the byssus gland. Magnified about 40 diameters.

photographed from the left side to show the area covered by, and the distribution of, the cementing material.

The use of the foot in creeping over surfaces has been described in a number of larval bivalves, and it is the chief means by which the young molluscs obtain foothold in a favorable locality.

Kellogg, '99, showed the importance of the foot in aiding the larva of *Mya* to obtain attachment. Sigerfoos, '07, noted that larval shipworms possess a powerful foot by means of which they creep rapidly over surfaces and select spots favorable for fixation to the exclusion of those which are not suitable. Field, '09, states that the larvæ of *Mytilus* creep from unfavorable situations upon seaweeds to locations which are better adapted to them. Belding, '10, describes the foot of the larval *Pecten*, a most important organ, since it is used for swimming as well as in crawling. Belding, '12, showed that in the full-grown larva of *Venus* the foot likewise is used in swimming as well as for crawling, and for procuring attachment.

That the use of the foot enables oyster larvæ to exercise some selection in seeking a place of attachment is evident from the wide differences in the number of spat procured when various kinds of cultch are placed together in the water. I have recently shown (Nelson, '23,) that oyster larvæ will not attach to shells which are extensively pitted by the boring sponge, *Clione*, or which are badly corroded and which present surfaces that are microscopically rough.

For some hours before oyster larvæ set they may be observed moving about over various surfaces with the velum extended, its cilia beating actively; and with the foot protruded and bent at right angles, thus bringing the distal half in contact with the substratum. The foot remains quiescent in this position while the larva slowly "skates" along, as it were, propelled by the velum, but aided somewhat by the cilia on the foot. Then, swimming upwards, the larva progresses a short distance through the water and drops down to the substratum to continue as before. In this manner the larva may wander over an appreciable area before it eventually attaches (Nelson, '21–A).

In this connection it is well to correct the idea held by oyster growers and by some scientists also, that attachment of the oyster larva occurs when its shell becomes so heavy that the animal sinks to the bottom, unable longer to swim. For example, Stafford ('13, 34) says: "when presumably the larvæ become too heavy to swim with ease, settle towards the bottom, creep about, and select some clean solid surface upon which they fix themselves."

That such is not the case must be evident to anyone who has observed extensive oyster sets on the bottoms of boats, or upon the bottoms of floats kept at the very surface of the water (Nelson, '17,). The full-grown oyster larva, so far from being helpless, is capable of more powerful swimming than at any time during its pelagic life. During the course of the development and growth of the larva the increase in size of the velum is more than commensurate with the increase in the weight of the shell. Plankton samples taken during the calm of early morning show large numbers of full-grown oyster larvæ swimming at the surface. At times the larva may project the foot into the surface film and, withdrawing the velum, may hang suspended by the foot and slowly rock the shell from side to side, much as in the familiar habit of pond snails hanging from the surface film.

Full-grown oyster larvæ are found mostly at the bottom because they develop during the last two days of their pelagic life a strong positive stereotropism, and since fixed objects are most abundant on the bottom the larvæ mainly congregate there. The intensity of attachment which occurs at the very surface of the water when suitable cultch is available, precludes the idea that increased weight of the shell or even positive geotropism are factors exclusively governing the time of fixation.

The use of the foot by oyster larvæ in crawling over surfaces is similar to that reported by Crozier, '21, who found that the adult *Lima* oriented itself away from the light by attaching the tip of the foot to the substratum after bending the free portion away from the source of illumination. The creeping of oyster larvae is also strikingly similar to that observed in the fresh-water Sphæridæ, and especially in the young of *Corneocyclas* when first liberated from the brood pouches of the parent (Nelson, '21–B).

In sessile forms, such as the oyster, which remain fixed on the spot where the larvæ set, keen competition follows when large numbers settle at one time. During heavy sets 1,000 larvæ may attach to one oyster shell 8 cm. long, upon an area which will permit not more than 6 oysters to grow to breeding age. It is significant that at such times of abundant settlement, when the water over the oyster beds may show as high as 250 full-grown larvæ per liter, the frequency of attachment per unit area of available cultch does not exceed a certain maximum.

Fig. 4 shows in a circle 6 mm. in diameter a group of 40 oyster larvæ just attached to an experimental shell. Unfortunately I have never witnessed the behavior of two oyster larvæ coming into contact with each other while circling about over a surface, but an examination of shells taken at times of maximum settlement indicates that the movements of the larvæ prior to final fixation not only aid them in finding a spot suitable for attachment, but that this behavior also tends to keep the larvæ separated from one another. The proximity of a neighboring larva could presumably be recognized only through touch, hence it is conceivable that here and there individuals failing to come in contact while circling about might attach close to one another, as occurred in six instances in Fig. 4. Examination of the most

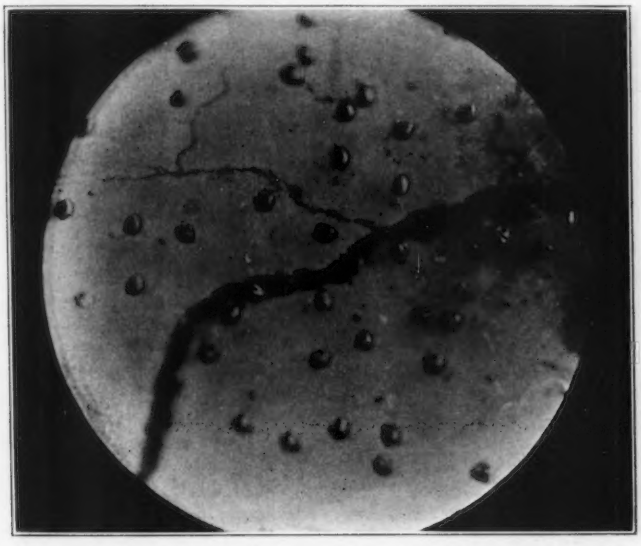

FIG. 4. Newly attached oyster larvæ on the surface of an oyster shell. Within this circle, 6 mm. in diameter, are 40 larvæ. Magnified about 15 diameters.

heavily set shells in our collection shows out of 500 newly attached larvæ only 57, or 11.4 per cent., which are within .5 mm. of another larva. This distance is taken since it represents the approximate radius of the circles described by the larvæ when first observed. Studies of the behavior of other bivalve larvæ

during the attachment period should give interesting information as to the prevalence of this type of behavior.

SUMMARY.

Full-grown larvæ of the oyster shortly before attachment move over an appreciable area of solid surface testing it out with the foot.

Actual attachment is preceded by circling movements, the larva finally coming to rest with the left valve held in contact with the substratum by the foot.

The suggestion of Stafford, 13, that the foot is the organ of attachment has been confirmed by direct observation. The use of the foot and of the mantle during fixation is here described.

The circling movements of the larva while crawling over a substratum not only aid it in obtaining a favorable foothold, but probably are also instrumental in producing a fairly even distribution of the spat.

CITATIONS.

Belding, D. L.

'10 A Report upon the Scallop Fishery of Massachusetts. The Commonwealth of Massachusetts, Boston.

'12 A Report upon the Oyster and Quahog Fisheries of Massachusetts. The Commonwealth of Massachusetts, Boston.

Brooks, W. K.

'79 Abstract of Observations upon the Artificial Fertilization of Oyster Eggs and the Embryology of the American Oyster. Am. Jour. Sci., New Haven, Vol. XVIII, pp. 425–427.

'80 The Development of the Oyster. Studies from the Biol. Lab. Johns Hopkins Univ. No. IV., pp. 1–106. Baltimore.

Crozier, W. J.

'21 Notes on Some Problems of Adaptation. 5. The Phototropism of *Lima*. BIOL. BUL., Vol. XVI., No. 2, p. 102.

Field, I. A.

'09 The Food Value of Sea Mussels. Bull. U. S. F. C., Vol. XXIX., p. 87.

Huxley, T. H.

'83 Oysters and the Oyster Question. Eng. Illus. Mag., Oct. and Nov., pp. 47–55, 112–121.

Jackson, R. T.

'88 The Development of the Oyster with Remarks on Allied Genera. Proc. Bost. Soc. Nat. Hist., Vol. XXIII., p. 531.

'90 The Phylogeny of the Pelecypoda. Mem. Bost. Soc. Nat. Hist., IV., p. 277.

Kellogg, J. L.

'99 Observations of the Life History of the Common Clam, *Mya arenaria*. Bull. U. S. F. C., 1899.

Nelson, J.

Annual Reports of the Department of Biology, N. J. Agr. Experiment Station, 1888–1893, 1900–1915. New Brunswick, N. J.

Nelson, T. C.

'17 Report of the Department of Biology for 1916.

'21–A Aids to Successful Oyster Culture. Part I., Procuring the Seed. Bulletin 351.

'21–B Report of the Department of Biology for 1920.

'23–B Report of the Department of Biology for 1922. N. J. Agr. Experiment Station, New Brunswick, N. J.

'23–A The Attachment of Oyster Larvæ. Abstr. in Anat. Rec., Vol. 24, No. 6, p. 395.

Ryder, J. A.

'82 Notes on the Breeding, Food, and Green Color of the Oyster. Bull. U. S. F. C., I., p. 403.

Sigerfoos, C. P.

'07 Natural History, Organization, and Late Development of the Teredinidæ, or Shipworms. Bull. U. S. F. C., Vol. XXVII., p. 193.

Stafford, J.

'10 The Larva and Spat of the Canadian Oyster. Am. Nat., Vol. XLIV., p. 343.

'13 The Canadian Oyster. Commission of Conservation, Ottawa.